

PCT

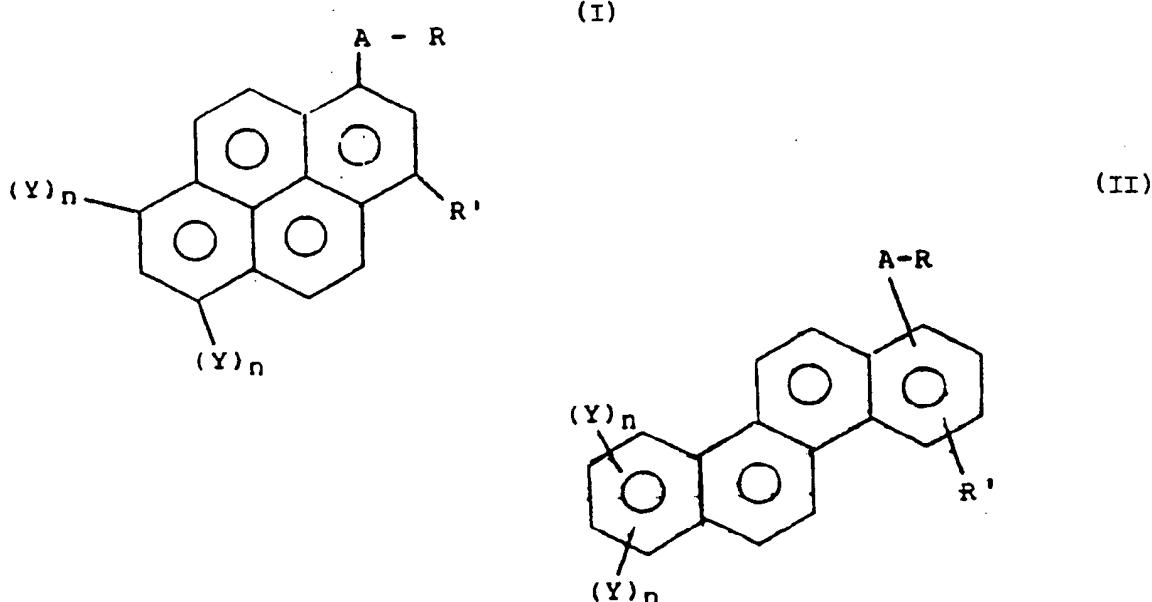
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/533, 33/50, 33/52 G01N 33/53, 33/532	A1	(11) International Publication Number: WO 90/05916 (43) International Publication Date: 31 May 1990 (31.05.90)
(21) International Application Number: PCT/US89/04828 (22) International Filing Date: 27 October 1989 (27.10.89)	Published <i>With international search report.</i>	
(30) Priority data: 271,161 14 November 1988 (14.11.88) US		
(71)(72) Applicant and Inventor: DOWBEN, Robert, M. [US/ US]; 7150 Eudora Drive, Dallas, TX 75230 (US).		
(74) Agents: SIGALOS, John, L. et al.; Sigalos, Levine & Mont- gomery, 2700 NCNB Center, Tower II, Dallas, TX 75201-3989 (US).		
(81) Designated States: CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, NL (European patent).		

(54) Title: FLUORESCENT IMMUNOASSAYS AND FLUORESCENT COMPOUNDS AND TRACERS THEREFOR



(57) Abstract

A polycyclic hydrocarbon of formulae (I) or (II), in which A is O, N, or S; R is H, a substituted or unsubstituted C₁-C₈ alkyl group, a substituted or unsubstituted C₂-C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or a halide with at least one Y being SO₃Z and Z is a halide; R' is H, Y or A-R; and n is 0 or 1 with at least one n equal to 1; which hydrocarbons can be coupled with a ligand or ligand-analog and utilized in fluorescent immunoassays.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT Austria	ES Spain	MG Madagascar
AU Australia	FI Finland	ML Mali
BB Barbados	FR France	MR Mauritania
BE Belgium	GA Gabon	MW Malawi
BF Burkina Fasso	GB United Kingdom	NL Netherlands
BG Bulgaria	HU Hungary	NO Norway
BJ Benin	IT Italy	RO Romania
BR Brazil	JP Japan	SD Sudan
CA Canada	KP Democratic People's Republic of Korea	SE Sweden
CF Central African Republic	KR Republic of Korea	SN Senegal
CG Congo	LI Liechtenstein	SU Soviet Union
CH Switzerland	LK Sri Lanka	TD Chad
CM Cameroon	LU Luxembourg	TG Togo
DE Germany, Federal Republic of	MC Monaco	US United States of America
DK Denmark		

FLUORESCENT IMMUNOASSAYS AND FLUORESCENT
COMPOUNDS AND TRACERS THEREFOREBACKGROUND OF THE INVENTION

This invention was made in part under United States Government Contract No. N00014-81-C-0619 and the United States Government has a non-exclusive, non-transferable, irrevocable, paid-up license to practice or have practiced for or on behalf of the United States, this invention throughout the world.

The present invention relates to fluorescence compounds and resultant tracers utilized as reagents in fluorescent immunoassay procedures to detect the presence of and/or quantify analytes in a mixture; even analytes that are present only in very low concentrations. In particular, the invention has relevance for the determination of constituents present in biological fluids such as blood serum, plasma, spinal fluid, urine, amniotic fluid, and the like and other fluids such as milk and water, or even solid materials such as wastes that can be solubilized. Such measurements are important for the practice of public health medicine as well as human and veterinary medicine. In addition, the determination of therapeutic drug levels and the presence of drugs of abuse in fluids is very valuable.

The determination of analytes which are pre-

sent only in very low concentrations has been a pervasive problem in analysis. The introduction of radioimmunoassays in 1956 enormously expanded the capabilities of clinical laboratories because 5 of the great sensitivity and selectivity of such radioimmunoassays, making possible the quantification of many analytes that could not easily be determined by other methods. They have been used to identify and quantify metabolites, hormones, drugs, and other biologically important substances both for purposes of diagnosis, and for purposes of assessing and following the effectiveness of medical treatment.

One type of immunoassay involves the competitive binding of an unknown quantity of analyte 15 in the sample to be analyzed, with a known quantity of analyte labeled by a tracer added to the reaction mixture, for a limited number of receptor-binding sites on specific antibodies that 20 combine with labeled and unlabeled analyte.

The use of radioactive tracers in immunoassays requires special licensing and special precautions in handling to minimize health hazards and contamination. Federal and state regulations that

must be followed in handling radioactive materials frequently impose an added burden in terms of time and effort required to perform the tests and to dispose of the used radioactive materials.

5 Standard curves constantly change owing to the decay of radioactivity and radiodecomposition of the constituent reagents. There are considerable batch-to-batch differences in manufacture. Furthermore, counters for the measurement of 10 radioactivity are large, expensive, cumbersome, and do not lend themselves to automation. Owing to these and other problems, immunoassays utilizing fluorescent tracers are gradually displacing the older assays using radioactive tracers.

15 However, only certain molecules exhibit the property of fluorescence to a significant extent. A fluorescent molecule will absorb a photon of light and utilize the energy to move an electron from the ground state to an orbital with a higher 20 energy level. This process is known as "excitation" and occurs at specific wave lengths of light. After a short interval of time; of the order of nanoseconds, the excited electron falls back into the ground state and emits a photon of

light. Some energy is lost in the process and the photon of emitted light is shifted toward the red part of the spectrum with respect to the wave length of exciting light. The difference between 5 wave lengths of exciting light and emitting light is known as the "Stokes shift".

The most commonly used fluorescent tracer in immunoassays is fluorescein. In the case of fluorescein, maximum excitation occurs when it is 10 illuminated with light of wave length 496 nm. The maximum emitted light occurs at a wave length of 516 nm. Thus, the Stokes shift is 20 nm. A number of other fluorescent tracers are known in the art and have been used in immunoassays, but 15 these fluorescent tracers have Stokes shifts of less than 35 nm. The use of tracers with Stokes shifts shorter than 35 nm presents a number of problems that limit fluorescence measurements.

First, fluorescence measurements are limited 20 by light scattering which causes part of the excitation light to be detected in the emission channel. In practice, light scattering interference is reduced by placing band pass optical interference filters in both the excitation and 25 emission channels. The greater the separation

between the wave length of maximum excitation and the wave length of maximum emission, and therefore of the band pass filters, the more effective such interference filters will be, and the more effective will be the minimization of light scattering background, an advantage not possible with tracers having Stokes shifts of 35 nm or below.

Also, serum and other biological fluids are intrinsically fluorescent. Almost all of the intrinsic fluorescence of biological fluids show Stokes shifts of less than 35 nm. Thus, utilization of fluorescent tracers with Stokes shifts of 35 nm or shorter allows more interference by the intrinsic fluorescence of serum and other biological fluids as well as other fluids to be tested.

Another limiting factor in fluorescence measurements of solutions is elastic Raman light scattering. In the case of aqueous solutions, Raman scattering occurs at approximately 300 wave numbers on either side of the frequency of the exciting light. Raman scattering is a problem with fluorescent tracers with small Stokes shifts.

Certain polycyclic compounds, in particular pyrene and chrysene compounds, have been shown to have relatively large Stokes shifts in purely

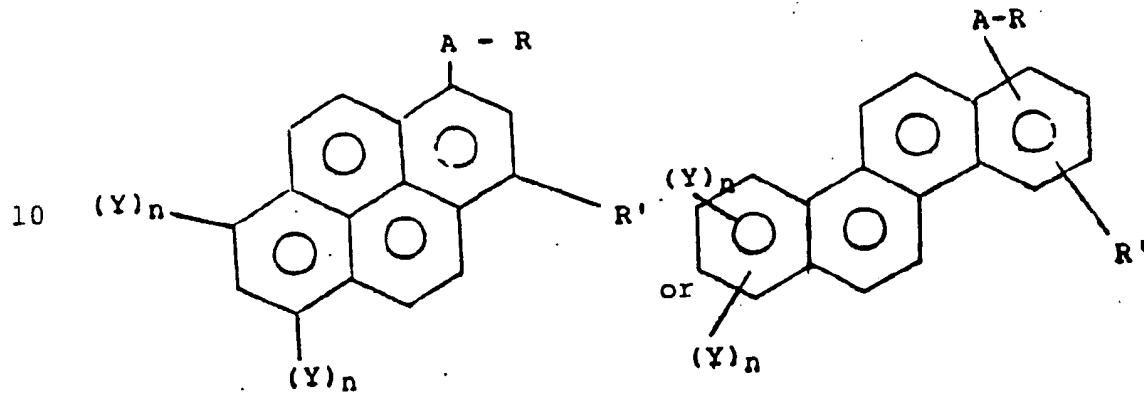
biochemical and cell biology research experiments. For example, 1-pyreneisothiocyanate is relatively nonfluorescent but reacts with the amine groups of antibodies to form conjugates with excitation 5 maxima of approximately 386 nm and emission maxima of 470 nm giving a Stokes shift of 84 nm. Similarly 6-chrysenemaleimide is a relatively nonfluorescent compound which forms adducts with the sulfhydryl groups of various compounds to form 10 fluorescent products with an excitation maximum of 360 nm and an emission maximum of 400 nm. 8-Hydroxypyrene-1,3,6-trisulfonic acid has been used as a pH indicator having an excitation maximum of 450 nm and an emission maximum of 515 nm 15 giving a Stokes shift of 65 nm. The various reports describing the use of such reactive pyrene or chrysene compounds fail to teach or suggest their use in immunoassay testing; whether fluorescence intensity or fluorescence polarization 20 is measured.

SUMMARY OF THE INVENTION

The present invention overcomes the noted problems of prior fluorescent compounds and tracers made therefrom in immunoassays and provides 25 tracers having large Stokes shifts thereby

enabling fluorescent assays of greater sensitivity with minimization of light scattering background, of interference by intrinsic fluorescence of the fluid being test, and of Raman light scattering.

5 Briefly, the present invention comprises
a polycyclic hydrocarbon



15

in which A is O, N, or S; R is H, a substituted or unsubstituted C₁ - C₈ alkyl group, a substituted or unsubstituted C₂ - C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or a halide, with at least one Y being SO₃Z and Z is a halide; R' is H, Y or A-R; and n is 0 or 1 with at least one n equal to 1.

The invention also comprises the tracers and immunoassay as hereinafter set forth.

25

DETAILED DESCRIPTION

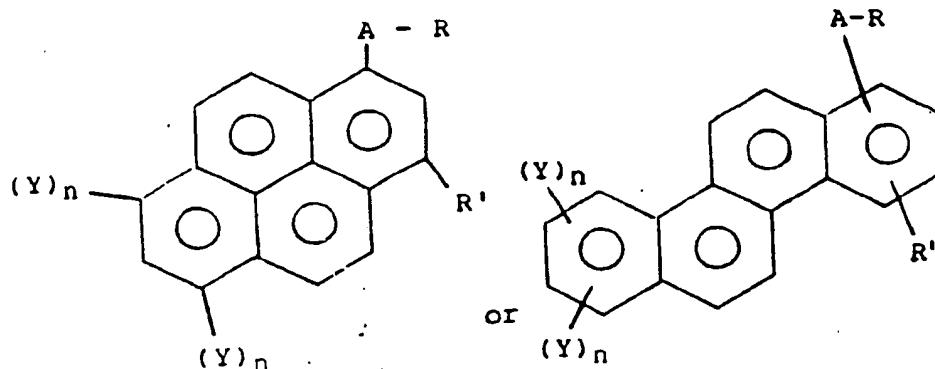
The terms "ligand" and "analyte" are used interchangeably herein and refer to constituents of fluids or dissolved materials; particularly biological fluids whose presence and/or concentration is to be measured. Antibodies that specifically bind to ligands can be raised in animals and such antisera isolated and purified by standard techniques well-known to those skilled in this art, or highly specific monoclonal antibodies can be selected and produced by again standard techniques. The ligands that can be identified and/or quantified by the assays of the present invention vary over a wide range of molecular weight and include, inter alia, such representative compounds as theophyllin, dilantin, phenobarbital, carbamazepine, gentamycin, streptomycin, amikacin, tobramycin, thyroxine, digoxin, digitoxin, procainamide, lidocaine, quinidine, primidone, propranolol, morphine, codeine, heroin, hormones, and the like.

Quantification of a ligand in a solution or mixture requires the preparation of a fluorescent-labeled ligand (tracer) that can be used to compete with unlabeled ligand for binding by a

specific antibody. The fluorescent compounds of the present invention can be bound to reactive amino, reactive carboxyl, or other reactive substituents present on the ligands or to ligand analogs possessing a reactive amino, reactive carboxyl reactive sulfhydryl, or other reactive groups to form suitable tracers. Some of the possible reactions that can be employed to link fluorescent tracers to ligands will be described below in the examples of embodiments of the invention and other known techniques can also be used for this purpose. The tracers formed by reacting the instant pyrene or chrysene compounds having the formula

15

20



in which A is O, N, or S; R is H, a substituted or unsubstituted C₁ - C₈ alkyl group, a substituted or unsubstituted C₂ - C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or 5 a halide, with at least one Y being SO₃Z and Z is a halide; R' is H, Y or A-R; and n is 0 or 1 with at least one n equal to 1.

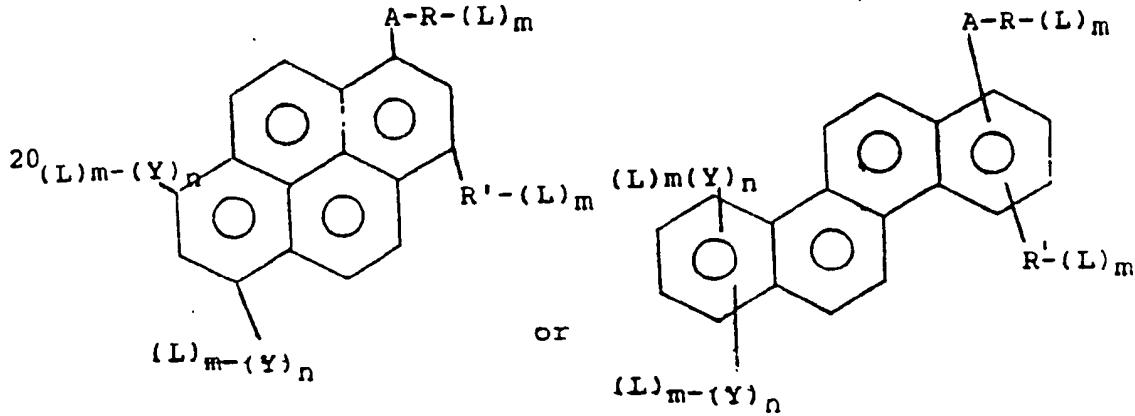
The pyrenesulfonic acid or pyrenesulfonyl chloride compounds are preferred with specific 10 preferred compounds being 8-acetoxy-1,3,6-pyrenetrisulfonyl chloride, 6-8-diacetoxy-1,3-pyrenedisulfonyl chloride, 8-ethoxy-1,3,6-pyrenetrisulfonyl chloride, 6,8-diethoxy-1,3-pyrenedisulfonyl chloride, and 15 8-succinylamino-1,3,6-pyrenetrisulfonic acid. As to the chrysene, 9-acetoxy-1,3,7-chrysenetrisulfonyl chloride is preferred, which form sulfonamides with reactive amino groups of a ligand or ligand analog.

20 In general, such compounds can be synthesized from pyrene and chrysene by the conventional process of forming the tetrasulfonic acid derivatives using anhydrous sodium sulfate and concentrated sulfuric acid. The hydroxy or amino 25 compounds are subsequently formed by reaction with

aqueous sodium hydroxide or ammonia at elevated temperatures under pressure. Reactive fluorescent derivatives are formed in one of two ways. The sulfonic acid groups are to be converted to 5 sulfonyl chlorides, for example, by the use of thionyl chloride. Sulfonyl chloride derivatives form adducts with reactive amino groups of ligands or ligand analog, for example. Alternatively, the ligand or ligand analog can be coupled to the 10 -O-R, -N-R, or -S-R group.

In general, tracer adducts of ligands or 15 ligand analogs with fluorescent moieties are formed by conventional procedures such as shown, for example, in U.S. Patents 3,996,345 or 4,420,568.

The pyrene and chrysene tracers of the present invention are of the formula



25 in which A is O, N, or S; R is H, a substituted or

unsubstituted C₁ - C₈ alkyl group, a substituted or unsubstituted C₂ - C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or a halide, with at least one Y being SO₃Z; R is H, Y, or A-R;

5 and n 0 or 1 with at least one n equal to 1; L is a ligand or ligand-analog; and m is 0 or 1 with only one m being 1.

One group of preferred fluorescent tracer compounds are based on pyrenesulfonic acids and

10 pyrenesylfonyl chlorides. Pyrene and chrysene compounds generally have large Stokes shifts, greater than 35 nm and often as large as 50 to 70 nm. A particular advantage of hydroxy, ethoxy, acetoxy or similarly substituted pyrenesulfonic acids is that their excitation is in the blue-green part of the spectrum, rather than the near ultraviolet part of the spectrum characteristic of other pyrene or chrysene compounds. Another advantage of these compounds is that they are readily

15 soluble in water, rather than being hydrophobic like other pyrene and chrysene compounds.

Fluoroimmunoassays can be carried out in several different ways. It is preferable to use a homogeneous assay method in which the antibody

20 bound ligand and tracer need not be separated from

the free ligand and tracer prior to measuring the fluorescence. Because only one incubation and no washings are required such assays can be performed simply and rapidly. Particularly useful are

5 fluorescence polarization immunoassays first described by Dandliker, W.B. et al., Immunochemistry 10, 219 (1973) and Spencer, et al. Clin. Chem. 19, 838-844 (1973). In such assays, the reaction mixture is excited with polarized

10 light, and the polarization of the fluorescence emission is measured. The degree of polarization of the fluorescence emission depends upon the ratio of free tracer to tracer bound to antibody, and thus, the ratio in unknown samples can be

15 determined by comparison to a standard curve. If owing to the high molecular weight of ligand, or to the low concentration of ligand, fluorescence polarization immunoassays are not sufficiently sensitive, another type of fluorescent immunoassay

20 may be employed such as those reviewed by Hemmila in Clin. Chem. 31, 359 (1985).

The invention will be further described in connection with the following examples which was set forth for purposes of illustration only.

A novel reactive fluorescent compound of great utility is 8-acetoxy-1,3,6-pyrenetrisulfonyl chloride which was synthesized as follows:

- 5 g 8-hydroxy-1,3,6-pyrenetrisulfonic acid
5 (available from Eastman Kodak) were refluxed in 50 ml acetic anhydride for 1.5 hrs. A light yellow solid precipitated that was collected by filtration, washed with ethylene chloride, and dried in a dessicator. 1.5 g of this material dissolved in
10 1 ml dimethylformamide was added to 10 ml thionyl chloride with constant stirring for 20 hrs in a fume hood. The mixture was then poured onto about 20 g crushed ice. After the vigorous reaction had subsided, the aqueous solution was transferred to
15 a separatory funnel and extracted three times with 20 ml portions of chloroform. The organic layers were combined and dried over anhydrous magnesium sulfate. The clear orange solution was filtered and the volume reduced to 15 ml by evaporation.
20 Hexane was added until cloudiness of the solution appeared. After storage at 2 degrees, golden brown crystals formed which were harvested. Yield = 31%. For steric reasons, only one molecule of ligand couples to each fluorescent probe molecule;
25 the remaining two sulfonyl chloride groups are

hydrolyzed.

To formulate an assay for phenobarbital, the fluorescent compound was reacted with 2-aminophenobarbital to form a tracer. Excitation maximum of this tracer was at 455 nm and emission maximum at 520 nm (Stokes shift 65 nm). The assay was performed as described by Dandliker, et al., supra.

A standard solution serum to be tested or other unknown was diluted 1:400 in 0.15 M phosphate buffer, pH 7.5, containing 0.01% sodium azide and 0.01% bovine gamma globulin. To 0.5 ml diluted sample was added 20 μ l phenobarbital tracer stock solution which contained 5 g/ml 2-aminophenobarbital in the above buffer. The best antibody and optimum antibody dilution was determined by generating antibody titration curves upon adding increasing amounts of antibody to a constant amount of tracer and determining the polarization using a fluorescence polarimeter. The appropriate amount of antibody in 20 μ l/buffer was added to the reaction and the mixture incubated at room temperature for 3 min. The polarization was then measured. Figure 1 illustrates a typical standard curve.

The polarization of unknown serum samples was

assessed in this manner and the concentration of phenobarbital calculated by comparison to the standard curve.

EXAMPLE 2

- 5 2.5g 1-nitropyrene was added with stirring over a period of 5 minutes to a mixture of 13g concentrated sulfuric acid and 3g anhydrous sodium sulfate at 60°C and stirred for an additional 15 min. breaking up the dark purple clumps that formed. Then 8 g fuming sulfuric acid was added over 30 min. at 60° with constant stirring. The whole mixture was added to 80g ice. An additional 60 ml water was added. After 18 hours the pH was adjusted to 5 and solution filtered. The aqueous phase containing 8-nitro-1,3,6-pyrenetrisulfonic acid was reduced with iron filings and acetic acid. The solution was filtered and 8-amino-1,3,6-pyrenetrisulfonic acid was precipitated by the addition of dilute HCl.
- 10 To 2.5 g of this product dissolved in 10 ml dry dimethyl formamide was added 0.75g succinic anhydride and the mixture stirred for 4 hrs. at 50° in the dark. 20 ml water was added and the mixture was slightly acidified and cooled on ice.
- 15 The precipitate was collected and the product

purified on preparative thin layer silica gel.

This fluorescent marker was utilized in an assay for thyroxine by forming an adduct with 3,5-dichloro-3',5'diiodothyroxine using dicyclohexylcarbodiimide. Excitation of the adduct tracer was 461 nm and the emission maximum was 524 nm (Stokes shift of 63 nm). This fluorescent thyroxine tracer was utilized in a fluorescence polarization assay similar to that described above.

EXAMPLE 3

To a mixture of 6.5g concentrated sulfuric acid and 1.5g anhydrous sodium sulfate was added 10g pyrene with stirring at 60° for 15 min. After 15 cooling to 50°, 4g fuming sulfuric acid was added in small portions over a 20 min. period. The dark reddish-black mass was cooled on ice. After standing for 2 hrs, it was broken up and dissolved in 70 ml water. After filtration, the product was 20 precipitated by saturating the filtrate with NaCl.

A mixture of 1.5g above product, 1.5g sodium hydroxide and 1.0 ml water was sealed in a glass pressure tube and heated at 155°C for 30 min; then at 165°C for 20 min.; and then at 170°C for 5 min. 25 The thin slurry thickened during this procedure.

The resulting mass was dissolved in water and the excess alkali neutralized. After filtration, the supernatant was acidified with acetic acid which precipitated the 6,8-dihydroxy-1,3-pyrenedisulfonic acid product. The diacetoxy derivative was first formed by treatment with acetic anhydride, and then the disulfonyl chloride formed as described in Example 1. Fig. 2 illustrates a typical standard curve.

A tracer adduct was formed by reacting the 6,8-diacetoxy-1,3 pyrenedisulfonyl chloride with 8-aminoethyltheophyllin. The tracer adduct had an excitation maximum of 464 nm and an emission maximum of 527 nm. The theophyllin tracer was utilized in a fluorescence polarization assay similar to that described in Example 1. The typical standard curve is shown in Figure 2.

EXAMPLE 4

To a mixture of 6.5g concentrated sulfuric acid and 1.5g anhydrous sodium sulfate was added 10g chrysene with stirring at 60° for 15 min. After cooling to 50°, 4g fuming sulfuric acid was added in small portions over a 20 min period. The dark mass was cooled on ice. After standing for 2 hrs it was broken up, and dissolved in 70 ml

water. After filtration, the product was precipitated by saturating the filtrate with NaCl.

The above product was mixed with 1.5g sodium hydroxide and 1.0 ml water, sealed in a glass pressure tube and heated at 155° for 30 min; then at 165° for 20 min; and then at 170° for 5 min. 5 The pressure tube was cooled and the resulting mass was dissolved in water and then brought to pH 9.0. After filtration, the supernatant was acidified with formic acid which precipitated the 7,9-dihydroxy-1,3-chrysenedisulfonic acid. The diacetoxyl derivative was first prepared by treatment with acetic anhydride, and then the disulfonyl chloride was formed by treatment with thionyl chloride as described in Example 1. 10 15

A tracer adduct was formed by reacting the 7,9-diacetoxyl-1,3-chrysenedisulfonyl chloride with diphenyl glycine. The tracer adduct had an absorption maximum of 438 nm and an emission maximum of 482 nm. The phenytoin tracer was utilized 20 in a fluorescence polarization assay similar to that described in Example 1.

EXAMPLE 5

8-Acetoxy-1,3,6-pyrenetrisulfonyl chloride was reacted with gentamycin to form a tracer adduct 25

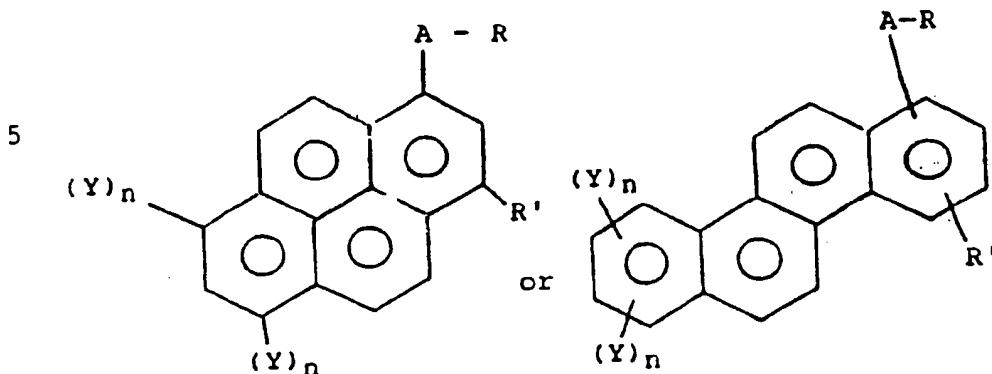
that was utilized in a fluorescence polarization assay similar to that described in Example 1 using excitation light of 455 nm and measuring the fluorescence at 520 nm. The average intrasample 5 correlation coefficients for six replications of 26 samples was 0.987. The average interassay correlation coefficients ($n = 26$) compared to commercially available gentamycin assays using a fluorescein based tracer was 0.966.

10 While the invention has been described in connection with preferred embodiments, it is not intended to limit the scope of the invention to the particular form set forth, but on the contrary, it is intended to cover such alternatives, modification, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

15

WHAT IS CLAIMED IS:

1. A polycyclic hydrocarbon of the formula



10 in which A is O, N, or S; R is H, a substituted or unsubstituted C₁ - C₈ alkyl group, a substituted or unsubstituted C₂ - C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or a halide, with at least one Y being SO₃Z and Z is a halide; R' is
15 H, Y or A-R; and n is 0 or 1 with at least one n equal to 1.

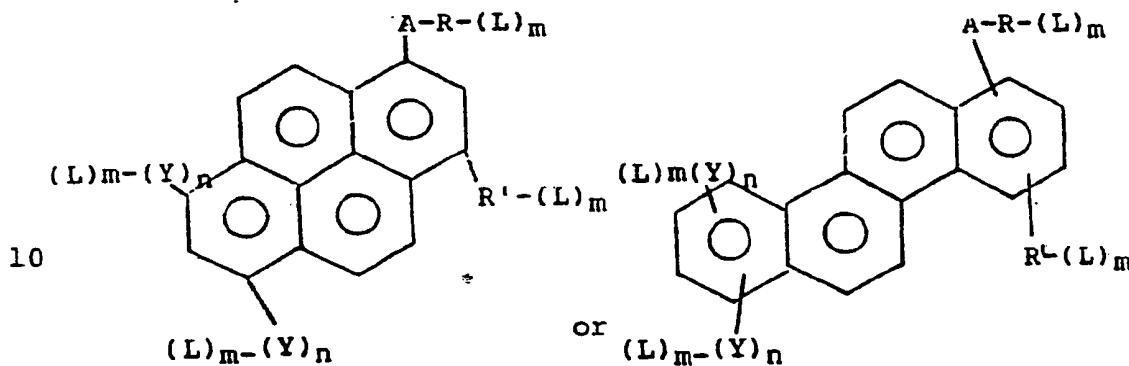
2. The hydrocarbon of claim 1 wherein the hydrocarbon is a pyrenesulfonic acid or pyrenesulfonyl chloride.

20 3. The hydrocarbon of claim 2 wherein the pyrene-sulfonic acid is 8-succinylamino-1,3,
6-pyrenetrisulfonic acid and the pyrenesulfonyl chloride is 8-acetoxy-1,3,6-pyrenetrisulfonyl
chloride, 6,8-diacetoxy-1,3-pyrenedisulfonyl
25

chloride, 8-ethoxy-1,3,6-pyrenetrisulfonyl chloride, or 6,8-diethoxy-1,3-pyrenedisulfonyl chloride.

4. 8-acetoxy-1,3,6-pyrenetrisulfonyl chloride.

5 5. A polycyclic hydrocarbon tracer of the formula



10 in which A is O, N, or S; R is H, a substituted or unsubstituted C₁ - C₈ alkyl group, a substituted or unsubstituted C₂ - C₈ ester group, or a substituted aryl group; Y is H or SO₃Z wherein Z is H or a halide, with at least one Y being SO₃Z; R' is H, Y, or A-R and n is 0 or 1 with at least one n equal to 1 and L is a ligand or ligand-analog; and m is 0 or 1 with only one m being 1.

15

20 6. The hydrocarbon tracer of claim 5 wherein the hydrocarbon portion is a pyrenesulfonic acid or pyrenesulfonamide.

25

7. The hydrocarbon tracer of claim 6 wherein the hydrocarbon portion is an 8-amidosuccinylamino

-1,3,6-trisulfonic acid, an 8-acetoxy-1-pyrene-sulfonamide-3,6,-disulfonic acid, 6,8-diacetoxy-1-pyrenesulfonamide-3-sulfonic acid, 8-ethoxy-1-pyrenesulfonamide-3,6,-disulfonic acid, or 6,8-5 diethoxy-1-pyrenesulphonamide-3-sulfonic acid.

8. The hydrocarbon tracer of claim 7 wherein the hydrocarbon portion is 8-acetoxy-1,3,6-pyrene-sulfonamide-3,6-disulfonic acid.

10 9. The hydrocarbon tracer of any one of claims 5 to 8 wherein L is a ligand-analog.

10. The hydrocarbon tracer of any one of claims 5 to 8 wherein L is a ligand.

15 11. A process for assaying a specimen to determine the presence and/or quantity of a ligand comprising admixing with said specimen an effect amount of a hydrocarbon tracer of claims 5, 6, 7, or 8 and an antibody specific to said ligand to be determined 20 and to the ligand-analog of said tracer, and subjecting said mixture to a homogeneous or heterogeneous fluorescent assay to determine the presence and/or quantity of said ligand in said specimen.

12. The method of claim 9 wherein the hydro-

carbon portion of said tracer is a pyrenesulfonic acid or pyrenesulfonamide.

13. The method of claim 10 wherein the hydro-
carbon portion is an 8-amidosuccinylamino
5 -1,3,6-trisulfonic acid, an 8-acetoxy-1-pyrene-
sulfonamide-3,6,-disulfonic acid, 6,8-diacetoxy-1-
pyrenesulfonamide-3-sulfonic acid, 8-ethoxy-1-
-pyrenesulfonamide-3,6,-disulfonic acid, or 6,8-
10 diethoxy-1-pyrenesulonamide-3-sulfonic acid.

14. The method of claim 11 wherein the hydro-
carbon portion is 8-acetoxy-1 pyrenesulfonamide
-3,6-disulfonic acid.

1/2

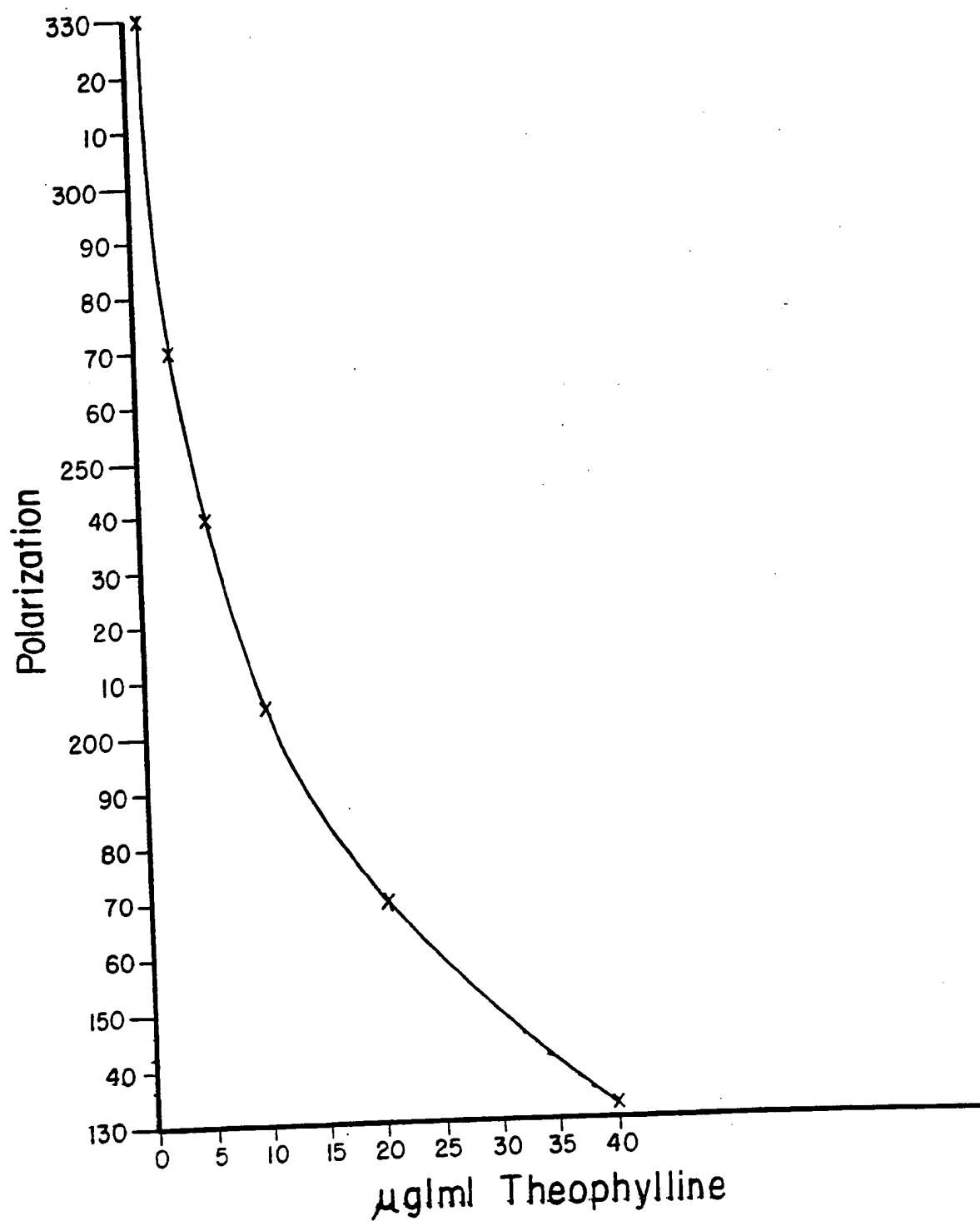


Fig. 1

SUBSTITUTE SHEET

2/2

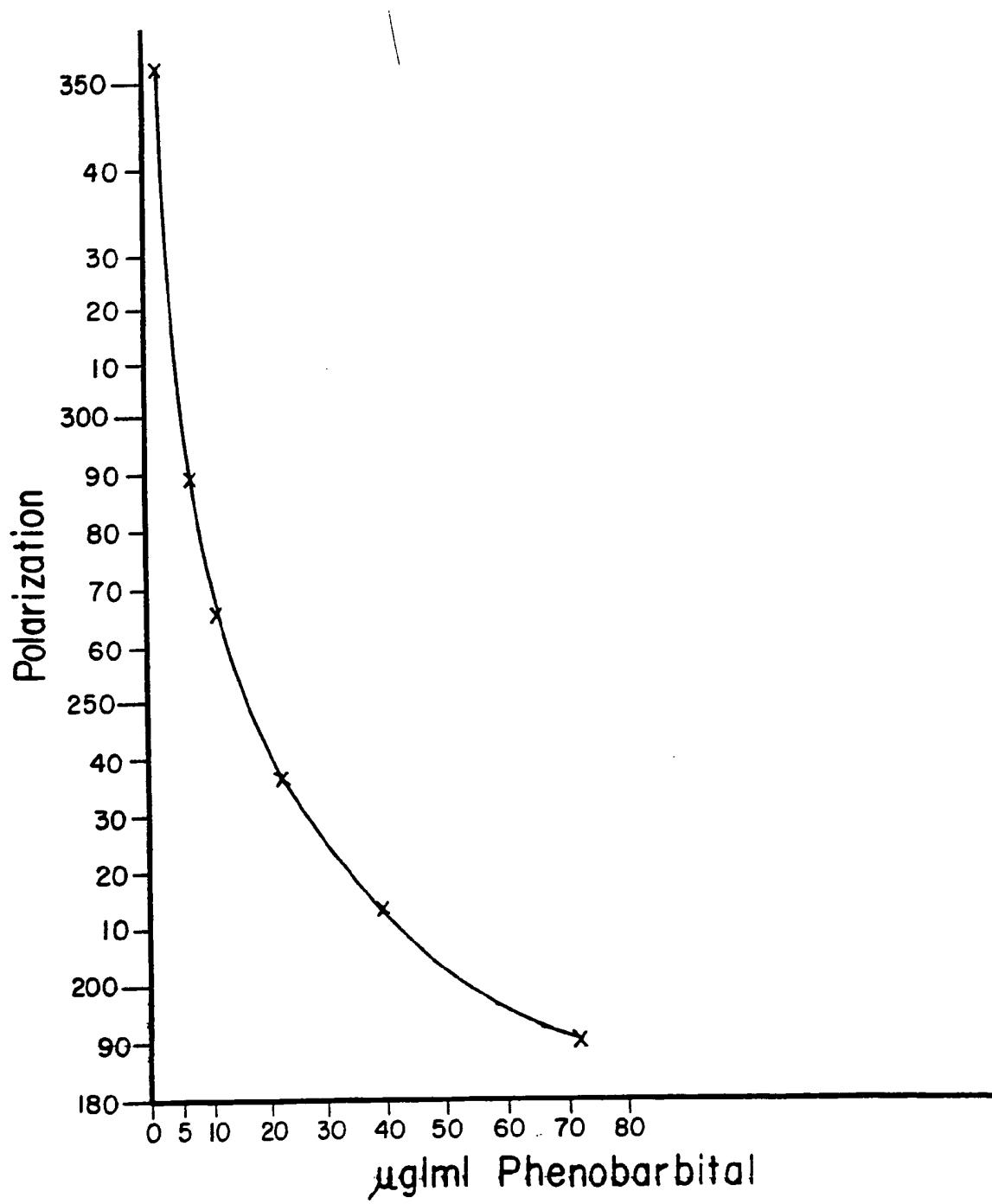


Fig. 2

CHARACTER SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04828

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) plus both National Classification and IPC
 IPC(5): G01N 33/533, 33/50, 33/52, 33/53, 33/532
 U.S. Cl. 436, 518, 546, 800, 805, 546/347

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched ¹	
	Classification Symbols	
U.S. Cl.	436/546, 518, 172, 800, 805, 435/125, 129, 546/347	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ²		

III. DOCUMENTS CONSIDERED TO BE RELEVANT ³

Category ⁴	Citation of Document, ⁵ with indication, where appropriate, of the relevant passages ⁶	Relevant to Claim No. ⁷
x y	AT,B, 385,755, Koller 10 May 1988 (See pages 1-4)	1-4, 6-8 5, 9-14
y	EP,A, 0278149 Krauth 17 August 1988 (See col. 8, line 57)	5, 9-14

¹ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "C" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "D" document relating to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

² later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

³ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

⁴ "T" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

⁵ "E" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

13 February 1990

Date of Mailing of this International Search Report

01 MAR 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

Daniel R. Passeri

PCT/ISA/210 (Rev. 08/86) (Form 11-67)

BEST AVAILABLE COPY

THIS PAGE BLANK (USPTO)